# Rapid Identification of Salamanders from the Jefferson Complex with Taxon-Specific Primers

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**The Jefferson complex comprises the Jefferson Salamander (*Ambystoma jeffersonianum*) and the Blue-spotted Salamander (*Ambystoma laterale*), in addition to unisexual salamanders, which present various combinations of the nuclear genome of the two bisexual species and show different levels of ploidy. The unisexuals arose by an ancestral hybridization event, and they all share a similar mitochondrial haplotype clearly different from that of the bisexual species. Although adults of *A. jeffersonianum* and *A. laterale* are usually easily differentiated morphologically, unisexuals can be difficult to identify visually because they may possess intermediate characters, or morphological traits from either of their bisexual counterparts. In the present study, we introduce a novel way to discriminate between bisexual and unisexual salamanders of the Jefferson complex based on taxon-specific primers designed in the mitochondrial cytochrome *b* region. This molecular approach allows for a simple, rapid, and non-invasive identification of unisexuals, using a single polymerase chain reaction (PCR) and small tissue samples that can easily be obtained from live specimens. We believe that this approach will be useful to screen a large number of individuals quickly in order to identify populations of the Jefferson Salamander, a crucial step towards the conservation of this threatened species.**

THE Jefferson Salamander (*Ambystoma jeffersonianum*) is distributed throughout regions of eastern North America. Locally isolated populations are restricted to places where suitable breeding ponds occur, and the species is considered threatened in Canada, and imperiled or vulnerable throughout most of its American range (Committee on the Status of Endangered Wildlife in Canada, 2000; NatureServe, 2006: http://www.natureserve.org/explorer). This salamander is part of the *Ambystoma* complex, along with three other bisexual species (*A. laterale* = Blue-spotted Salamander, *A. texanum* = Small-mouthed Salamander, and *A. tigrinum* = Tiger Salamander) and arrays of unisexual salamanders, which are almost exclusively female (Bogart and Klemens, 1997). Those unisexuals are of different ploidy levels, containing at least one copy of the *A. laterale* nuclear genome and various combinations of the genome from the other three bisexual species. To designate the genomic composition of salamanders within the *Ambystoma* complex, an informal system was adopted (Lowcock et al., 1987) in which each letter identifies a set of chromosomes (J = *A. jeffersonianum*, L = *A. laterale*, T = *A. texanum*, and Ti = *A. tigrinum*). Twenty different genomic unisexual compositions have been uncovered to date, from diploids (LJ, LT) to pentaploids (LLLLJ). Interestingly, all unisexuals share a similar mitochondrial haplotype clearly different from that of the bisexual species and that is related to the Streamside Salamander (*Ambystoma barbouri*; Hedges et al., 1992; Bogart, 2003). Thus, it has been postulated that unisexual salamanders arose by an ancestral hybridization event involving an ancestor of *A. barbouri* (Robertson et al., 2006).

Within the *Ambystoma* complex, the smaller Jefferson complex comprises *A. jeffersonianum* and *A. laterale*, in addition to unisexuals containing various combinations of the nuclear genome of the two bisexual species (LJ, LLJ, LJJ, LLLJ, LJJJ, LLJJ, LLLLJ; Bogart, 2003). As unisexuals reproduce by gynogenesis or hybridogenesis, they depend on males from the bisexual species for reproduction, and are thus always found in sympatry with either *A. jeffersonianum* or *A. laterale* (Bogart et al., 1989). Although the bisexual adult males can easily be differentiated morphologically from each other and from unisexuals, the unisexuals are difficult to discriminate because they may possess intermediate characters, and often they cannot be differentiated from either one of the bisexual species (Petranka, 1998).

Various identification methods have been developed to discriminate members of the Jefferson complex. These include allozyme electrophoresis (Bogart, 1982; Bogart et al., 1985), chromosome counts (Sessions, 1982), blood erythrocyte size (Uzzell, 1964; Wilbur, 1976; Austin and Bogart, 1982), DNA content by flow cytometry (Lowcock et al., 1991), and microsatellite markers (Julian et al., 2003; Ramsden et al., 2005). However, all techniques except microsatellites require invasive sampling and cannot be applied to preserved tissues or eggs. Most techniques also need costly equipment. In this paper, we introduce a simple, rapid, and non-invasive method to identify salamanders of the Jefferson complex using taxon-specific primers to be used in a single polymerase chain reaction (PCR).

## MATERIALS AND METHODS

To design specific primers, we targeted the 307 base pairs (bp) of DNA sequence from the mitochondrial cytochrome *b* gene used by Hedges et al. (1992). We retrieved 29 Jefferson complex sequences from GenBank, including six from *A. jeffersonianum*, ten from *A. laterale*, and 13 from unisexuals (accession numbers Z11615–Z11624, Z11627–Z11628, Z11630–Z11632, Z11635, Z11641, Z11643–Z11646, Z11651–Z11652, Z11655–Z11660) distributed among two Canadian

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**Table 1.** Sampling Locality of *Ambystoma laterale* (LL) and Unisexuals Collected in the Field from Québec, Canada, and Results of Molecular Identification with Specific Primers.

| Location | Coordinates (NAD83) | Year | Sample type | *n* | Primers ID |
|---|---|---|---|---|---|
| Mont Saint-Hilaire | 45°33′N, 73°08′W | 1999 | Larva | 6 | Unisexual |
| Carignan (site 1) | 45°23′N, 73°20′W | 2004 | Tail tip | 1 | Unisexual |
| Carignan (site 2) | 45°26′N, 73°21′W | 2004 | Tail tip | 1 | LL |
| Carignan (Île Goyer) | 45°28′N, 73°16′W | 2004 | Tail tip | 1 | LL |
| Mont Royal | 45°30′N, 73°35′W | 2004 | Tail tip | 60 | Unisexual (57) LL (3) |
| Collines d'Oka | 45°29′N, 74°04′W | 2005 | Egg | 1 | Unisexual |
| Mont Mégantic | 45°25′N, 71°07′W | 2005 | Tail tip | 1 | Unisexual |
| Mont Rougemont | 45°29′N, 73°02′W | 2005 | Tail tip | 1 | Unisexual |

provinces (Ontario and Prince Edward Island) and five American states (Connecticut, Illinois, Maine, New York, Vermont). By focusing on sites that discriminate unisexuals from bisexual species, we designed a reverse primer (Hybrid-REV 5′–CAA ATR AAA AAG AAT GAG–3′) that is specific to all genomic combinations of unisexuals from the Jefferson complex. When used in conjunction with the primer Universal-FW (5′–ACA GCA GAC ACA TCA TCA–3′), designed to anneal to DNA from all members of the Jefferson complex, a 113-bp fragment is amplified in unisexuals only. To make sure that the absence of this unisexual band is not due to PCR failure, a 258-bp fragment is also amplified in all specimens using the forward primer Universal-FW and a modification of the reverse primer originally designed by Hedges et al. (1992): Universal-REV (5′–CTC AAA AAG ATA TTT GTC CTC A–3′). Consequently, *A. jeffersonianum* and *A. laterale* should only display the 258-bp fragment, whereas unisexuals should additionally display the specific 113-bp band.

To assess the efficiency of the developed primers, we tested them on frozen museum samples from different localities previously identified by allozymes, flow cytometry, or morphology. Additionally, we analyzed 72 unknown specimens from deciduous or mixed deciduous–conifer forests sampled from 1999–2005 in eight localities in southern Québec, Canada (Table 1). Tail tips, eggs, or larvae were collected in the field, and tissue was stored in 95% ethanol. DNA extractions were performed with Quick Lysis (Olsen et al., 1996) for all specimens, except museum samples for which a standard phenol–chloroform protocol was used (Sambrook et al., 1989).

We carried out multiplex-PCR amplification in a total volume of 20 µl. Each reaction mixture was composed of 2 µl 10× buffer (100 mM Tris-HCl, 0.5 mM $MgCl_2$, 1% Triton X-100, 500 mM KCl), 0.5 mM of each dNTPs, 32 mM of $MgCl_2$, 2 pmol of the forward universal primer, 4 pmol of the unisexual specific reverse primer, 12 pmol of the universal reverse primer, 1 U of *Taq* Polymerase, and approximately 50 ng of template DNA. The PCR profile was an initial denaturation of 2 min at 94°C, followed by 32 cycles of 1 min at 94°C, 90 sec at 50°C and 90 sec at 72°C, with a final extension of 10 min at 72°C. PCR products were subjected to electrophoresis on a 3% agarose gel, stained with Vistra Green (Amersham Biosciences), and visualized by UV transillumination. We determined the size of the fragments by comparison to a 100-bp ladder.

## RESULTS

The amplified fragments were of expected sizes for most museum specimens; all displayed the universal 258-bp fragment, whereas unisexuals additionally displayed the specific 113-bp band. However, one sample (ROM 17575) from Whiteshell Provincial Park (Manitoba) that was originally designated as *A. laterale* based on morphological criteria was identified as a unisexual using this assay. The sequencing of this specimen (accession number DQ490106) using the primers of Hedges et al. (1992) confirmed it as a unisexual. Most of the field specimens were identified as unisexuals. In the Mont Royal population, where a large number of salamanders were sampled, 95% of the individuals were unisexuals.

## DISCUSSION

Specific primers represent an increasingly popular method to discriminate sister taxa or cryptic species and have also proven useful for the detection of identification errors (Lin et al., 2002; Noël et al., 2004; Tessier et al., 2004). The specific primers developed in this study allow for the quick identification of *Ambystoma* salamanders from the Jefferson complex. Altough alternative approaches such as direct sequencing of PCR products, microsatellites, allozyme electrophoresis, or chromosome data may also be used to identify particular genotypes or obtain population genetics information, specific primers present the advantage of being simpler, cheaper, and rapid when only the distinction between unisexuals and bisexuals is needed. Although the distribution of *A. jeffersonianum* is not fully known, the species seems to have a patchy distribution and suitable habitat is threatened by urban encroachment. We believe that this non-invasive approach will be useful to quickly screen a large number of individuals to accurately identify populations of *A. jeffersonianum*, a crucial step towards the conservation of this threatened species.

The specific primers developed in this study also allowed us to uncover the presence of unisexuals where only *A. laterale* were thought to occur. In Québec, a unisexual from Mont Mégantic was identified, which represents a slight extension to the known range of unisexuals in this province (Petranka, 1998). A specimen from Manitoba that was previously identified morphologically as *A. laterale* was also identified as a unisexual. As the currently known distribution of LLJ unisexuals is limited to Wisconsin in the west (Petranka, 1998), this result could mean that the range of these unisexuals is larger than previously thought. However, two additional museum specimens sampled from the same locality were identified as *A. laterale*. Although it is probable that the two forms co-exist in this area, unisexuals usually outnumber bisexual individuals at a particular site (Lowcock et al., 1991), as observed in the Mont Royal population in

Québec. Therefore, the apparent presence of a unisexual in the Whiteshell Provincial Park may also be explained by a labeling error. Additional sampling from this area is thus needed to ascertain the presence of LLJ unisexuals in Manitoba.

## MATERIAL EXAMINED

Institutional abbreviations follow Leviton et al. (1985), except for BOG which represents catalogue numbers from J. Bogart (University of Guelph). The method used to identify the specimen is indicated in parentheses: A = allozymes, F = flow cytometry, and M = morphology.

*Ambystoma jeffersonianum*. BOG 10539 (A), Adams County, Ohio; BOG 10542 (A), Adams County, Ohio; ROM 16811 (A), Halton County, Ontario; AMNH 129341 (A), Hamden County, Massachusetts; AMNH 129343 (A), Hamden County, Massachusetts; BOG 29574 (A), Litchfield County, Connecticut; BOG 29487 (A), Oswego County, New York; BOG 29460 (A), Sullivan County, New York; BOG 19765 (A), Wentworth County, Ontario; BOG 29508 (A), York County, Pennsylvania.

*Ambystoma laterale*. ROM 20363 (A), Cochrane County, Ontario; ROM LL579 (A), Ozaukee County, Wisconsin; ROM 17029 (M), Whiteshell Provincial Park, Manitoba; ROM 17574 (M), Whiteshell Provincial Park, Manitoba; ROM 17575 (M), Whiteshell Provincial Park, Manitoba.

Unisexual *Ambystoma*. ROM 17660 (F), Haliburton County, Ontario; ROM 17662 (F), Haliburton County, Ontario; ROM 17666 (F), Haliburton County, Ontario; ROM 18685 (A), Peel County, Ontario.

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